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ISOLATION AND PURIFICATION OF BROMELAIN ENZYME FROM PINEAPPLE JUICE AND STUDY CYTOTOXIC ACTIVITY AGAINST CANCER CELLS

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Abstract

The work included the isolation of Bromelain enzyme from pineapple juice using different biochemical techniques which included: ammonium sulfate precipitation, diagnosis and gel filtration chromatography on Sephadex G-100. It was found that only the second peak had a high activity for Bromelain, the apparent molecular weight of the isolated Bromelain using gel filtration chromatography and SDS-PAGE was (27000) and (24000) Dalton respectively, this study was led to distinguish the cytotoxic action of purified enzyme Bromelain from pineapple juice remove on human breast cancer (MCF-7) cells in vitro contrasted with human hepatic (WRL-68) cells utilizing MTT measure. Charted 6 crystal program incorporating non-straight relapse with one-way ANOVA followed by Dennett's different comparison test to get information addressing the mean+ SD. The outcomes uncovered high poisonousness of the concentrate against MCF-7 cells with IC50 (194.5µg/ml) and showed high-decreased action toward WRL-68 cells with high IC50 esteem while the least feasibility (26.389 ±3.73022) showed up in MCF. 7 cells under treatment with 400 µg.ml-1 contrasted and high practicality in WRL-68 cells. The review reasoned that has a significant wellspring of anticancer medications with protected and particular action on disease cells in contrast with human typical cells.

Keywords: Pineapple Plant, Gel Filtration, Breast Cell Cancer Line, Enzyme Activity Test On Cancer Cells, Liver Cell Line.

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Introduction

Fruit Bromelain is a cysteine protease accumulated in pineapple plant ,which is one of most abundant in the plant ,it has large activity in application in food industry ,the investigation of partial purification and isolation of the enzyme have been began since 1849[1],this enzyme is the major chemically since 1876 in the plant [2],and was identified in the beginning by Marcano in 1891 [3],Bromalin is present in all parts of the plant that has systematic name as (EC3.4.22.33),also is a monomer of glycosylated enzyme which has high a proleolytic activity against peptide bond of portions such as milk and meat [4-5],Bromelain enzyme has wide application in medicine [6], has great benefits and is used in many treatments such as infections ,allergies and anticoagulants [7,8],there are a lot of studies that showed the effect of the enzyme on cancer cells [9],few research have noticed the potential role inhibitory in cancer cell growth in Breast cancer in women , For this study, the effect of the purified bromelain enzyme from plant juice on breast cancer cell lines compared with human hepatocytes was examined by enhancing and defining the importance of this enzyme as an anti-cancer cell killing agent. This study is considered the first of its kind in the country.

2.MATERIALS AND METHODS

2.1. Obtainment of pineapple juice:

The pineapple fruit was purchased from the local market in Kirkuk city, Iraq the plant product of Costa Rica from Del Mont .company, its juice was prepared at room temperature and pressure, using a pulped mass of 650 g that initially was to simple filtration through cotton to remove the dispersed solids. Phosphate buffer at pH 7.0 and 7.5 was used. The solution volume was adjusted to 1.0 L. The concentrate contains the same attributes found in the writing and was involved as references [10,11,12]

2.3.determination of Bromelain enzyme activity:

The enzyme activity was carried out as follows by a method (13)and modified by (14) :activity of any enzyme can be assayed either by substrate or product formation method , in this assay ,casein acts as substrate ,the amount of tyrosine is liberated along with other amino acid with incubation period 20min,and measured as an absorbance by the activity of Bromalein at 280 nm are compared to a standard curve ,Bromalein activity was measured as units per ml (U/ml)and tyrosine a standard .as shown in diagram below In blank Bromalein (RB) which have all the reagent expect the enzyme while the RM have the substrate after adding the enzyme ,also the blank which have all the reagent without enzyme and substrate , Calculating the enzymatic activity of bromelain enzyme The standard curve of the amino acid tyrosine was used if the amount of tyrosine released from the enzymatic reaction was measured by projecting the absorbance onto the standard curve of tyrosine, which was prepared using different concentrations of the original solution of tyrosine (1 mg per 100 ml) and the enzymatic unit is the amount of enzyme Which releases a micromole of tyrosine per unit minute , As shown in Figure(1)The enzymatic activity was calculated by using the equation as below.

The enzyme activity was calculated using the following equation for U/ml per ml of enzyme

Micrograms of each released tyrosine multiplied by the dilution factor divided by the volume of enzyme in ml multiplied by the time measured in minutes

The amount of released tyrosine is calculated from the absorbance at 280 nm and is equal to the absorbance of the reaction mixture - the plank absorbance.

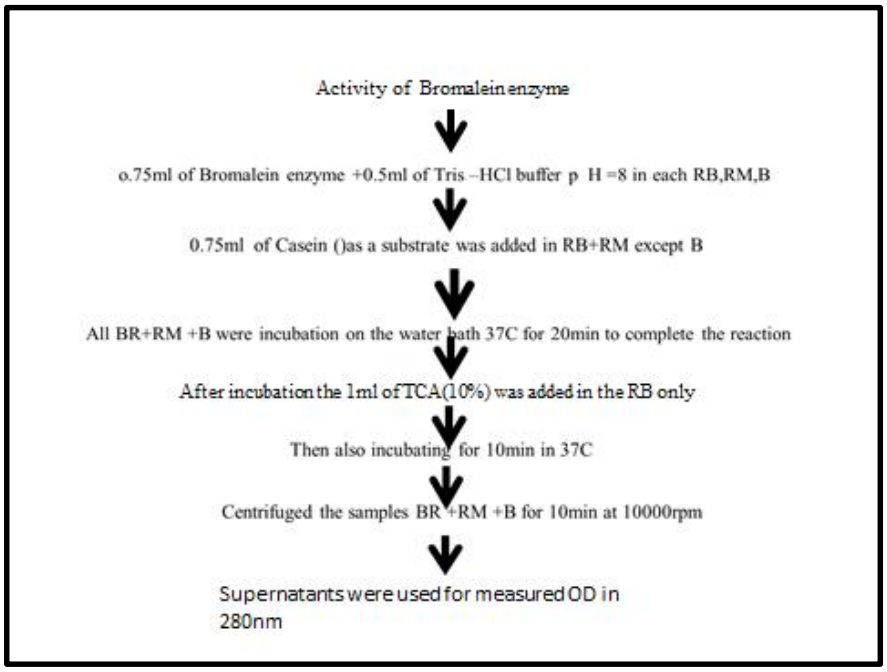

Activity of Bromalein enzyme
o.75ml of Bromalein enzyme +0.5ml of Tris –HCl buffer p H =8 in each RB,RM,B
0.75ml of Casein ()as a substrate was added in RB+RM except B
All BR+RM +B were incubation on the water bath 37C for 20min to complete the reaction
After incubation the 1ml of TCA(10%) was added in the RB only
Then also incubating for 10min in 37C
Centrifuged the samples BR +RM +B for 10min at 10000rpm
Supernatants were used for measured OD in 280nm

Diagram of assay Bromalein enzyme

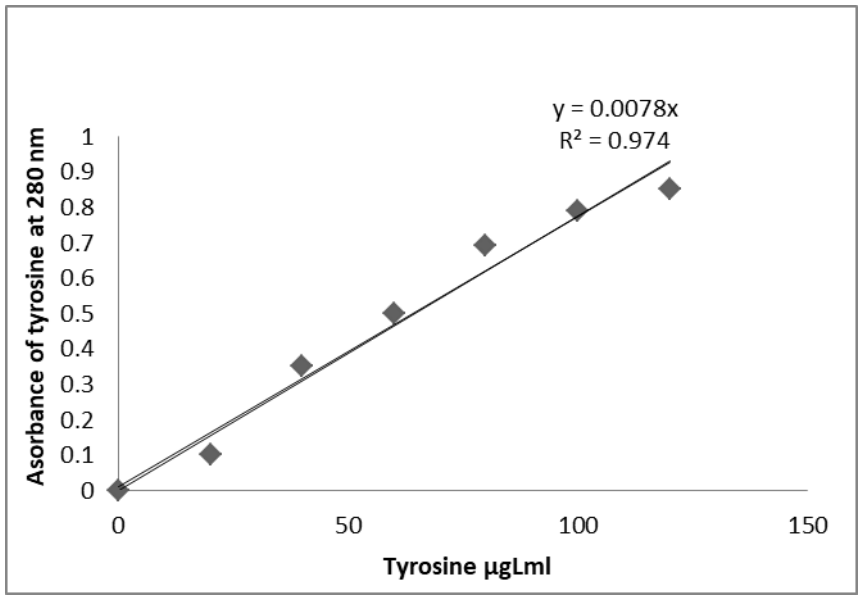


Figure (1) Standard curve for the amino acid tyrosine

2.4.Ammonium sulfate precipitation:

Bromelain precipitation was performed utilizing ammonium sulfate immersion, the supernatant was carried to 70% immersion with ammonium sulfate by leisurely adding strong ammonium sulfate. the option of ammonium sulfate was slow, so a modest quantity was added and permitted to break down before making further options. the blend was mixed electrically at (4) C for (60) min, then left for the time being in the cooler. the hasten framed was then isolated by centrifugation at (6000xg)for 20 min(19). .protein fixation was assessed and Bromelain was not set in stone. the protein in accelerating and supernatant are resolved to utilize the altered lowery (20).

2.5. Dialysis

The dialysis sac containing the suspension in (step 1)was dialyzed against 0.01M of ammonium carbonate. the solution was stirred with a magnetic stirrer overnight at 4 C the buffer was changed during dialysis (21).than the bromelain in the supernatant solution containing the enzyme was estimated by modified lowery (20) and the Bromelain activity is determined (2) -3) than stored for the next step.

2.6. Gel Filtration Chromatography

In the present study, the column a dimension of 2×100 cm which contained a gel Sephadex G-100 to a height of (90)cm the exclusion limit for this type of the gel is 100000 dalton (21) or the molecular weight ranges for peptides and globular proteins of (5000-100000) Dalton .depending on the volume of this column which 450 ml, it was packed with a slurry of the gel in buffer Tris –HCl p H 8. The slurry was carefully poured down on a glass rod to prevent air bubbles from forming. A concentrated sample (5ml) of the proteinaceous material, which was prepared in (2-5), was applied to the top of the bed of Sephadex G-100, followed by Tris –HCl buffer. Elution of proteinaceous materials was carried at a flow rate (24)ml/hour with a definite time interval of (10)min, using Tris –HCl buffer p H 8, as an Eluant. the fractions were collected using a fraction collector. The proteinaceous compounds in each fraction collected were detected by following the absorbance at wavelength (280 nm) using a UV-Visible Spectrophotometer .peak was combined separately in each fraction (22).

2.7. Freeze-Dryer (Lyophililization)Technique

The enzyme fraction which was obtained from gel filtration was dried using a freeze–dryer (lyophilization)technique to obtain power or a concentrated protein. the enzyme was kept in a deep freeze at (-20)Co in a tight tube to be used in further investigation.

2.8. Electrophoresis

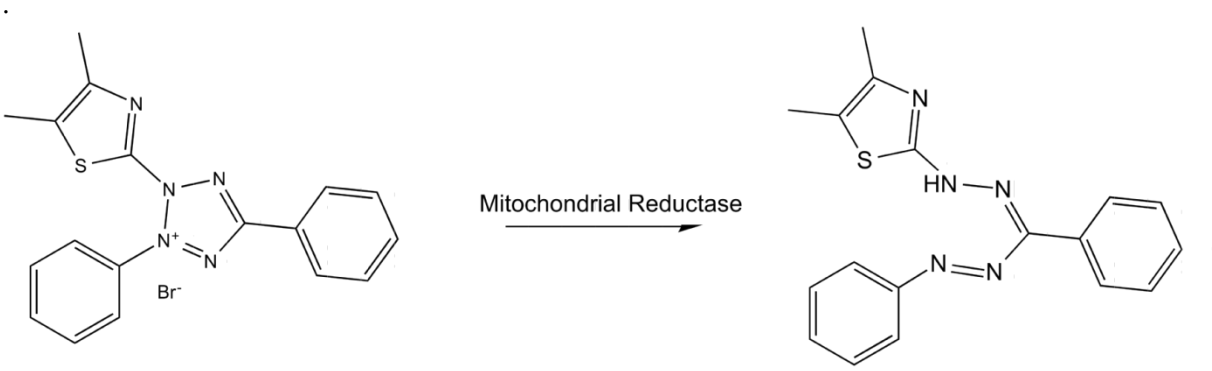
Only one sample can be run per each tube ,(2-7)which was applied on sodium dodecyl sulfate polyacrylamide Gel Electrophoresis (SDS-PAGE)using disc electrophoresis unit fit instrumentation (23).

2.9. Toxicity assay of purified bromelain from pineapple juice

Cell lines were obtained to study the toxicity of its natural and breast cancer types, which are due to human cells, and the standard kit for measuring toxicity was also obtained from Sigma-Aldrich Company.

2.10. MTT (Methyl Thiazolyl Tetrazolium) Cytotoxicity Assay:

The prepared cell lines are placed in the measuring plate, which consists of 96 holes, then 20 µl of the sterilized toxicity assay solution is added. This solution contains yellow tetrazolium, which is dissolved in physiological saline solution at a concentration of 5 mg per ml. Then incubated for two hours, a dimethyl sulfoxide solution is added to dissolve the violet Informazione, then its absorbance is measured at 570 nm by ELISA reader. As shown in the equation below The measurement is repeated three times to reduce the error rate in the measure.



3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
(MTT)

(*E*,*Z*)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan
(Formazan)

2.12. statistical analysis

ANOVA was used to analyze the obtained data and the adjusted representation in addition to the standard deviation, and through regression analysis, the effective concentration (the effective value of the purified enzyme) was calculated.

3-Results and Discussion

3.1.precipitation of the protein (Bromelain)

Bromelain has been purified, from pineapple fruit, it was isolated from pineapple juice. The most commonly used is ammonium sulfate, which has a high water solubility the result predicted that 70% saturation with ammonium sulfate to crude preparation (after homogenizing the pineapple juice) produced maximum protein precipitate This result was by Devakate et al. have independently utilized salt precipitation and ionic trade chromatography to extricate Bromelain from explained pineapple juice. The last method prompted a 10-overlay advancement in bromelain and gave a threefold better return than salt precipitation [15]. A bromelain readiness with similar enzymatic action has been gotten by Costa et al. utilizing a mix of cation trade and size prohibition chromatography [16]. Hernández et al. have too tried the mix of cation trade and size prohibition chromatography, and this approach brought about the recuperation of 41.15% of enzymatic action, relative to the underlying stem extricate [17]. Fondness chromatography is one more fruitful method for Bromelain extraction (18) recovering approximately 80% of Bromelain from the pineapple fruit by ammonium sulfate in the same range of saturation. After the precipitation, the precipitate was dialyzed to remove the small molecule compounds.

3.2.Dialysis

As shown in table (1), the specific activity was slightly increased after dialysis this might be due to the removal of the small molecules and increasing the purification of Bromelain.

3-3-Gel Filtration:

This strategy was applied to isolate the proteinous materials, which were acquired by ammonium sulfate precipitation technique and diagnosis for rough planning from pineapple squeeze, the aftereffects of elution displayed in figure (2)indicated that these were chiefly two proteinous compounds, and B was getting with high Bromelain movement (2.56U/ml), which top A with exceptionally low Bromelain action (2.3U/ml) which was dismissed, The particular action of chemical was higher in top B (1.6U/mg)fold the action in unrefined as displayed in a table (1).

Table (1):partial purification steps of Bromalien from pineapple juice

Fraction or step	Volume ml	Protein mg/ml	Total protein (mg/ml)	Activity U/ml	Total activity (U/mg)	S.P.Activity (U/mg)	Purification fold	Yield of original activity
Initial extract	50	0.5	25	0.11	5.5	0.22	1	100
$(NH_4)_2SO_4$	25	0.6	15	0.13	3.25	0.21	0.6	59
Dialysis	23	0.45	10.23	0.2	4.6	0.45	2	80.6
G-100-Sephadex peak A	10	0.19	1.9	0.23	2.3	1.2	5.45	42
Peak B	8	0.2	1.6	0.32	2.56	1.6	7.2	46.5

However, these results obtained agree with many researchers in estimating the partial weight of this enzyme, as the average partial weight ranges between 22-27kDa, and this according to varies to the sources in which the enzyme was purified and isolated from the plant and other factors such As fruit ripening, size, harvest period and climatic conditions of the producing country[19-22].

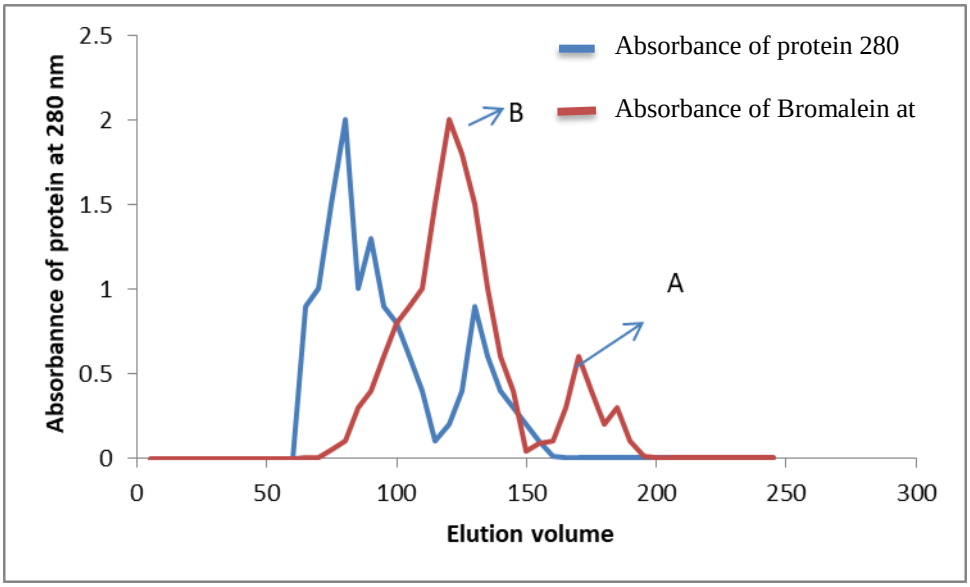


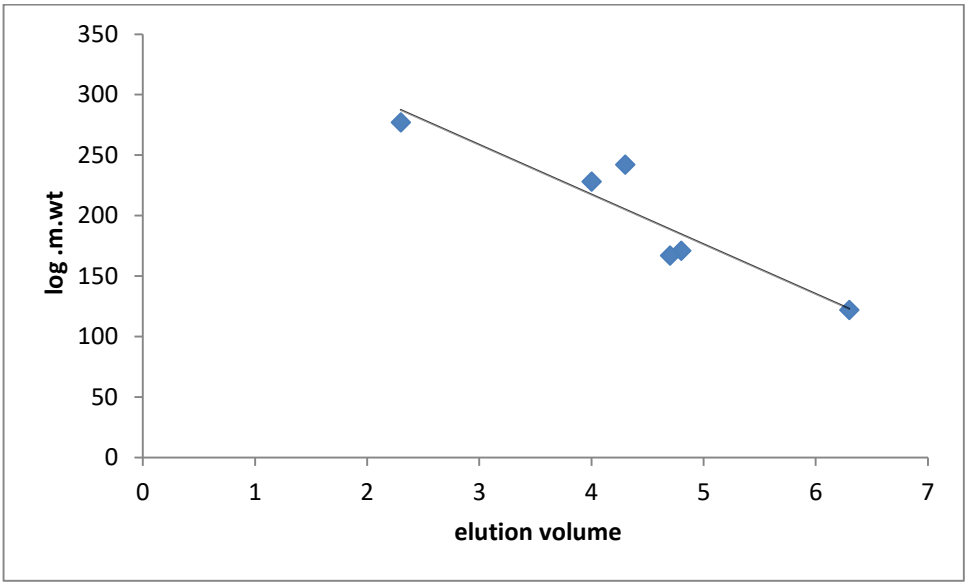
Fig (2):Elution profile of Bromelian enzyme on Sephadex G -100

3.4. Molecular Weight determination of Bromelain by GEL Filtration

The partial weight of the enzyme bromelain was calculated, which was obtained from the highly effective and purified package using gel filtration and using the same column as well as standard proteins as shown in Table (2) From pineapple juice was found to be (25±500Da)by gel filtration chromatography .

Table 2: shows the standard curvature of standard proteins with known partial weight with Gel –filtration

Materials	Molecular Weight (Dalton)	Elution volume
Blue dextran	2000000	122
BSA	67000	171
α-Amylase	58000	167
Papain	23000	242
Tryptophan	204	277
Unknown (peak b)	24000	228



Fig(3): A plot of the logarithm molecular weight of known proteins versus elution volumes on a Sephadex G-100

A plot of a logarithmic load of portentous material versus the elution volumes demonstrated in table (2) gives a straight line as delineated in figure(3). The molecular weight of the unknown protein nous compound isolated by a similar section chromatography as displayed in (not entirely set in stone from the standard bend, which was addressed by figure (3). Wellspring of Bromelain is roughly equivalent to (24,000)dalton .this finding was in great concurrence with the previous outcomes where it was accounted for that the sub-atomic load of Bromelain was ((24.5-32.5) Dalton from of pineapple plant [23- 27].

3.5. Moulecular weight determination by SDS –PAGE

The electrophoretic mobility of Bromelain in SDS gels was determined.the enzyme migrated as a single band in control only as shown in figure (3) with an apparent molecular weight of (26000) Dalton which was determined by using known molecular weight compounds as shown in figure (4)

Fig(4): shows SDS gel electrophorese of the standard protein with purified Bromelain the tubes from left to right contained (25)µg of standard protein employed to calibrate the columns were :a. Bromelain purified b. Urease (M.wt.480000)c. Egg albumin (M.wt.45000).Pepsin (M.WT.36000).e. Bovin serum albumin (BSA) 67000.

3.6. MTT Assay

The results obtained in the table (3) showed that the five concentrations used for the purified enzyme gave a variation of results between diseased and healthy cell lines and that this enzyme had a direct effect on breast cancer cells at 194.5 µg/ml to reduce cells .

Table 3 shows the practical application of a breast cancer cell line in comparison with normal hepatocytes using M±SD.

Purified Bromelain enzyme µg/ml	MCF-7	WRL-68
400	26.389±3.7022	80.637±4.11
200	45.079±1.9122	87.492±1.541
100	65.646±2.263	90.484±0.54
50	79.54±3.132	93.518±0.53
25	92.196±3.425	95.023±0.722

We also found the lowest breast cancer viability from purified Bromelain concentration of 400µg/ml was (26.399±3.7022), it increases (92.196±3.425)with reducing the concentration to(25 µg/ml)table (3)and figure (5) .also found the highest of human haptic cells was 94.023±0.722 with 25 µg/ml concentration. the lowest viability was 80.631±4.1172 which was observed at 400 µg/ml (table 3 and Figure 5).

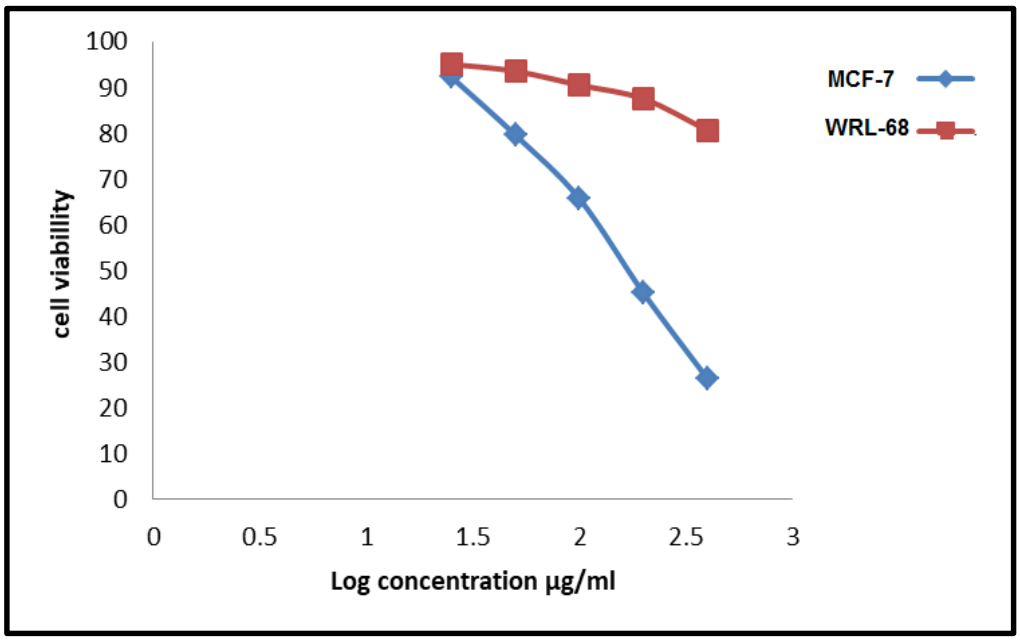


Figure (5) percentage of the practicality of human malignant under purified Bromelain

Compared With Normal Cell

Many studies deal with the effectiveness of the bromelain enzyme and its ability to inhibit the growth of cancer cells. The effectiveness of this enzyme is because it can stone the immune system, that is, white blood cells and the formation of monocytes and phagocytes inhibits the rapid growth of cancer cells. It was also found that it affects only diseased cells and does not affect This is consistent with studies (28-32).

The conclusion The Bromelain enzyme is one of the degrading enzymes found in all parts of the plant in and this study we used pineapple juice and isolated the bromelain enzyme the reason for choosing this enzyme was its high importance in many aspects of life, especially with medication to treat cancerous diseases if many cancer cells and that it can be used as an alternative treatment for chemotherapy and the doses that are used in the treatment of this disease, especially in our country.

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